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Growth Factor-Induced Tyrosine Phosphorylation of Hrs, a Novel 115-Kilodalton Protein with a Structurally Conserved Putative Zinc Finger Domain

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The activation of growth factor receptor tyrosine kinases leads to tyrosine phosphorylation of many intracellular proteins which are thought to play crucial roles in growth factor signaling pathways. We previously showed that tyrosine phosphorylation of a 115-kDa protein is rapidly induced in cells treated with hepatocyte growth factor. To clarify the structure and possible function of the 115-kDa protein (designated Hrs for hepatocyte growth factor-regulated tyrosine kinase substrate), we purified this protein from B16-F1 mouse melanoma cells by anti-phosphotyrosine immunoaffinity chromatography and determined its partial amino acid sequences. On the basis of the amino acid sequences, we molecularly cloned the cDNA for mouse Hrs. The nucleotide sequence of the cDNA revealed that Hrs is a novel 775-amino-acid protein with a putative zinc finger domain that is structurally conserved in several other proteins. This protein also contained a proline-rich region and a proline- and glutamine-rich region. The expression of Hrs mRNA was detected in all adult mouse tissues tested and also in embryos. To analyze the Hrs cDNA product, we prepared a polyclonal antibody against bacterially expressed Hrs. Using this antibody, we showed by subcellular fractionation that Hrs is localized to the cytoplasm; we also showed that that tyrosine phosphorylation of Hrs is induced in cells treated with epidermal growth factor or platelet-derived growth factor. These results suggest that Hrs plays a unique and important role in the signaling pathway of growth factors.

Peptide growth factors exert pleiotropic biological effects on a variety of cell types (9). These effects include mitogenesis, enhanced motility, differentiation, metabolism, and morphogenesis. Signals for all these effects are mediated through specific receptors expressed on the cell surface. Growth factor receptors possess intrinsic tyrosine kinase activity in the cytoplasmic domains, and the binding of growth factors to the receptors enhances the kinase activity, leading to tyrosine phosphorylation of many intracellular proteins in addition to the receptors themselves (65, 70). Many of these tyrosine kinase substrates, such as phospholipase C-γ (37, 39), ras GTPase-activating protein (25, 27), the p85 regulatory subunit of phosphatidylinositol 3′-kinase (7, 26, 66), Shc (52), Nck (36, 48), and Src family tyrosine kinases (8, 42), possess Src homology 2 (SH2) domains and bind tightly to the phosphotyrosine-containing sequences in the autophosphorylated receptors through their SH2 domains (4, 31). On the other hand, proteins that do not have an SH2 domain are also tyrosine phosphorylated in growth factor-stimulated cells. They include c-Raf serine/threonine kinase (43), mitogen-activated protein kinase (56), insulin receptor substrate-1 (64), and several proteins identified by the use of anti-phosphotyrosine antibody affinity chromatography, such as the Src substrate p120 (24), eps8 (11), and eps15 (12). Thus, the growth factor signaling pathways are considered to involve the tyrosine phosphorylation of both SH2 domain-containing proteins and other signaling molecules.

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Hepatocyte growth factor (HGF), also known as scatter factor, has several distinct biological effects on its various target cells. It has mitogenic activity for hepatocytes in primary culture, as well as other cultured cells (16, 23, 57). It dissociates epithelial cell colonies into individual cells (14, 62, 67) and increases the motility of epithelial cells (61). It also induces epithelial tubulogenesis (41) and angiogenesis (3, 18). The c-*met* proto-oncogene product (c-Met receptor) has been identified as the receptor for HGF (2, 45). It belongs to the cell surface receptor tyrosine kinase family (49), and all the activities of HGF are mediated through the cytoplasmic domain of the c-Met receptor (15, 32, 55, 68). Like other growth factors, HGF induces the tyrosine phosphorylation of several intracellular proteins (5, 19, 46, 53). It is shown that tyrosine phosphorylation of some of these molecules, such as phosphatidylinositol 3′-kinase, phospholipase C-γ, growth factor receptor-binding protein 2 (GRB-2), and c-Src, or their association with the autophosphorylated c-Met receptor is required for signal transduction of some cellular responses (53, 72). However, it is not known whether tyrosine phosphorylation of only these molecules is sufficient for signal transduction of all the biological actions of HGF.

We previously showed that tyrosine phosphorylation of a 115-kDa protein was induced in B16-F1 mouse melanoma cells treated with HGF (33). Phosphorylation of this protein was not detected in cells transfected with c-Met receptors that were mutated in the cytoplasmic domain and could not mediate the biological responses (33). These results suggest that the 115-kDa protein (designated Hrs for HGF-regulated tyrosine kinase substrate) plays an important role in the intracellular signaling triggered by HGF.

In this study, to clarify the structure and possible function of Hrs, we purified Hrs from B16-F1 cells and cloned its cDNA. The nucleotide sequence of the cDNA revealed that Hrs is a

novel putative zinc finger protein with a proline-rich region, as well as a proline- and glutamine-rich region. We also showed that Hrs is a cytoplasmic protein and that its tyrosine phosphorylation is also induced in cells treated with epidermal growth factor or platelet-derived growth factor. These results suggest that Hrs has a unique and important function in growth factor signaling.

MATERIALS AND METHODS

Growth factors, antibodies, and cell culture. Recombinant human HGF was provided by the Research Center, Mitsubishi Kasei Corp. (63). Recombinant human epidermal growth factor and platelet-derived growth factor BB were purchased from Wakunaga Co. and Boehringer Mannheim, respectively. Anti-phosphotyrosine antibody PY20 and anti-hemagglutinin (HA) epitope antibody 12CA5 were obtained from ICN Biomedicals and Boehringer Mannheim, respectively. Anti-Met antiserum was raised against the chemically synthesized C-terminal 21-amino-acid peptide of mouse c-Met receptor as described previously (34), and anti-cyclic AMP-responsive element-binding protein (anti-CREB) antiserum, which was raised against the C-terminal 16-amino-acid peptide of human CREB, was provided by M. Yoshida. All the cell lines used in this study were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Purification of Hrs. Hrs was purified from B16-F1 cells by a combination of immunoaffinity chromatography with antiphosphotyrosine antibody PY20 and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Near-confluent cells in a 150-mm-diameter dish were incubated with 100 ng of HGF per ml for 5 min at 37°C, washed twice with phosphate-buffered saline containing 0.01% EDTA and 0.2 mM Na_3VO_4, and lysed in 3 ml of lysis buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, and a mixture of phosphatase and protease inhibitors [1 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin per ml, 1 μg of leupeptin per ml, and 1.4 μg of pepstatin A per ml]). Cell lysates from 30 dishes were mixed and centrifuged at 105,000 × *g* for 1 h. The supernatant was incubated at 4°C for 16 h with gentle agitation with 100 μg of PY20 bound noncovalently to 1 ml of protein A-Sepharose (Pharmacia). The Sepharose beads were packed in a column, washed with 25 ml of lysis buffer, and further washed with 25 ml of washing buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.013% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid [CHAPS], 1 mM EDTA, and the mixture of phosphatase and protease inhibitors). Tyrosine-phosphorylated proteins bound to the Sepharose were eluted with 5 ml of washing buffer containing 40 mM sodium phenylphosphate. This purification procedure was repeated five times, and tyrosine-phosphorylated proteins recovered from a total of 150 dishes were concentrated by ultrafiltration (Centricon 30; Amicon) and separated by preparative SDS-PAGE (5% polyacrylamide) under reducing conditions. The gel was stained with Coomassie brilliant blue, and Hrs was excised and recovered from the gel by electroelution.

Partial amino acid sequence analysis of Hrs. Purified Hrs was concentrated to a final volume of 50 μl by ultrafiltration and then digested with 10 ng of lysylendopeptidase (Wako). The generated peptide fragments were separated by reverse-phase high-performance liquid chromatography (SMART system μRPC C2/C18 SC2.1/10 column; Pharmacia) and sequenced with an Applied Biosystems model 476A protein sequencer.

cDNA cloning of Hrs. Total RNA was prepared from B16-F1 cells by cesium chloride centrifugation, and poly(A)$^+$ RNA was purified by oligo(dT) affinity chromatography. The primers, 5′-GT(GATC)GT(GATC)CA(GA)GA(TC)AC(GATC)TA-3′ and 5′-TT(TC)TC(GAT)AT(GATC)CC(GA)AA(TC)TT-3′, were chemically synthesized. By using these two primers and the poly(A)$^+$ RNA as a template, DNA fragments were amplified by reverse transcriptase-PCR and a 246-bp fragment was obtained. To construct a cDNA library, cDNA was synthesized from B16-F1 poly(A)$^+$ RNA by using the Time Saver cDNA synthesis kit (Pharmacia) with random primers, and cDNAs with *Eco*RI adaptors at both ends were ligated to the *Eco*RI-digested λZAP II vector. This library was screened first by the 246-bp PCR product, and 4 positive clones of 8×10^5 independent clones were identified. Since the clones did not cover the entire coding sequence, the library was further screened with the cloned cDNAs to obtain full-length cDNA.

Northern blotting. Mouse adult and embryo multiple-tissue Northern (RNA) blot membranes were purchased from Clontech, and hybridization was performed as specified by the manufacturer with a 629-bp *Eco*RI-*Sca*I fragment (nucleotides 1 to 629) of Hrs cDNA as a probe.

Preparation of antibody against bacterially expressed Hrs. The whole insert of cDNA clone 5-1 with *Eco*RI linkers at both ends was cloned in frame into the *Eco*RI site of the pGEX-2T vector (Pharmacia), and a fusion protein with glutathione-*S*-transferase was expressed in *Escherichia coli* JM109. As the fusion protein was not solubilized by sonication, the insoluble fraction was lysed with boiling SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 5% SDS, 10% glycerol, 0.0025% bromophenol blue, 5% 2-mercaptoethanol) and the fusion protein was purified by two rounds of preparative SDS-PAGE (6% polyacrylamide). Rabbits were immunized with the purified fusion protein, and antiserum (anti-Hrs) was raised.

Immunoprecipitation and immunoblotting. Near-confluent cells in 90-mm-diameter dishes were lysed with 1 ml of lysis buffer and centrifuged at 105,000 × *g* for 30 min. The supernatant was incubated for 16 h at 4°C under gentle agitation with 7.5 μl of anti-Hrs, 7.5 μl of anti-Met, 2 μg of PY20, or 2 μg of 12CA5 that were bound to 10 μl of protein A-Sepharose. After the Sepharose beads were washed three times with 1 ml of 10 mM Tris-HCl (pH 7.4)–150 mM NaCl–0.1% Nonidet P-40–1 mM Na_3VO_4, the immunoprecipitated proteins were eluted with 50 μl of boiling SDS sample buffer and separated by SDS-PAGE (7.5% polyacrylamide) under reducing conditions. The proteins were transferred to a polyvinylidene difluoride membrane (Trans-Blot; Bio-Rad) and incubated with anti-Hrs (1:100), PY20 (1 μg/ml), or anti-Met (1:100) for 2 h; this was followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactive proteins were visualized with the enhanced chemiluminescence (ECL) Western blotting (immunoblotting) detection system (Amersham).

Construction and transfection of HA-tagged Hrs cDNA. To construct a full-length Hrs cDNA, *Eco*RI-*Sca*I (nucleotides 1 to 629), *Sca*I-*Xho*I (nucleotides 630 to 1396), *Xho*I-*Sph*I (nucleotides 1397 to 1898), and *Sph*I-*Eco*RI (nucleotides 1899 to 2458) fragments were ligated in order into the *Eco*RI site of pBluescript. To clone the full-length cDNA into the mammalian expression vector, pCG-HA, which carries the sequence for the influenza virus HA epitope (21) (a gift from J. Fujisawa), two primers were synthesized: 5′-CTATCTA GAATGGGGCGAGGCAGCGGCAC-3′ and 5′-CTTCTCAATGCCGAACT TGG-3′. Using these primers and Hrs cDNA as a template, a 630-bp fragment was amplified. This fragment was digested with *Xba*I and *Bst*PI, and the resultant 531-bp fragment, which contained a partial Hrs sequence (nucleotides 43 to 567, amino acids 1 to 176) with an *Xba*I linker just upstream of the translation initiation codon, was ligated to the *Xba*I-*Hin*dIII site of pCG-HA together with the *Bst*PI-*Hin*dIII fragment of Hrs cDNA (nucleotides 568 to 2458; the *Hin*dIII site is derived from pBluescript). This expression vector, pCG-HA-Hrs, was transfected into Cos-7 cells by the standard calcium phosphate precipitation method. Two days later, the cells were lysed and analyzed.

Subcellular fractionation. Near-confluent cells in a 90-mm dish were incubated with or without HGF and homogenized in 1 ml of homogenizing buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, and the mixture of phosphatase and protease inhibitors) with a Teflon homogenizer. The nuclear fraction was precipitated by centrifugation at 1,000 × *g*, and the pellet was incubated in 250 μl of homogenizing buffer containing 0.4 M NaCl for 1 h on ice. After centrifugation at 105,000 × *g* for 1 h, the supernatant (crude nuclear extract) was recovered. The postnuclear supernatant was centrifuged at 105,000 × *g* for 1 h, and the cytoplasmic fraction (supernatant) and membrane fraction (pellet) were separated. The membrane fraction was solubilized by incubation in 1 ml of homogenizing buffer containing 0.5% Nonidet P-40 for 1 h on ice and was centrifuged at 105,000 × *g* for 1 h; the supernatant (membrane protein) was collected. The cytoplasmic and membrane protein fractions, as well as the crude nuclear extract, were immunoprecipitated with 7.5 μl of anti-Hrs or anti-Met, and the immunoprecipitates were analyzed by immunoblotting. Otherwise, the proteins of each fraction were directly immunoblotted by anti-CREB (1:200).

Nucleotide sequence accession number. The GenBank/EMBL/DDBJ accession number of the mouse Hrs sequence is D50050.

RESULTS

Purification and partial amino acid sequence analysis of Hrs. When B16-F1 cells were incubated with HGF for 5 min, tyrosine phosphorylation of a cellular protein with an apparent molecular mass of 115 kDa (designated Hrs), as well as a minor 110-kDa protein, was detected by immunoprecipitation and immunoblotting with the anti-phosphotyrosine antibody PY20 (33) (Fig. 1A). Under these conditions, in which the cells were cultured with 10% fetal calf serum, the basal tyrosine phosphorylation level of c-Met receptor was relatively high compared with that in the cells cultured with 0.5% fetal calf serum (32, 33) and the increase in its phosphorylation level was not evident in HGF-treated cells (Fig. 1). However, the electrophoretic mobility shift of c-Met receptor, which was probably caused by the change in the tyrosine phosphorylation state, was induced upon HGF treatment (Fig. 1).

Hrs did not react with antibodies against several known tyrosine-phosphorylated proteins (data not shown), implying that Hrs is a novel protein or a protein that is not known to be tyrosine phosphorylated. When the same samples used for immunoblotting were stained with silver, a band corresponding to Hrs was clearly detected (Fig. 1B). We thus purified Hrs by

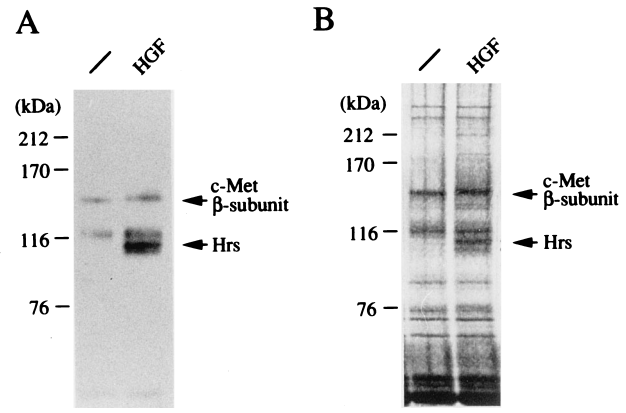

FIG. 1. Tyrosine phosphorylation of Hrs in B16-F1 cells treated with HGF. Cells were incubated without (−) or with 100 ng of HGF per ml for 5 min at 37°C, lysed, and immunoprecipitated with PY20. The immunoprecipitates were separated by SDS-PAGE under reducing conditions and immunoblotted with PY20 (A) or stained with silver (B). Molecular mass standards are indicated in kilodaltons.

PY20 immunoaffinity chromatography. B16-F1 cells from 150 dishes (approximately 2.5×10^9 cells) were incubated with HGF for 5 min, lysed, and applied to a PY20 immunoaffinity column. Tyrosine-phosphorylated proteins that had bound to the column were eluted with phenylphosphate, concentrated, and separated by SDS-PAGE. After the gel was stained with Coomassie brilliant blue, Hrs was excised and recovered from the gel by electroelution. Silver staining of the purified protein after SDS-PAGE revealed a single band (data not shown). The recovery of Hrs was roughly 3 to 5 μg from about 2.5×10^9 cells, judging from the density of the silver-stained band. To determine the partial amino acid sequences of Hrs, the purified protein was digested with lysylendopeptidase and the resulting peptide fragments were separated by reverse-phase high-performance liquid chromatography. Six partial sequences were determined (Fig. 2B). None of the peptide sequences matched those in the Swiss Prot or NBRF protein sequence databases, indicating that Hrs is a novel protein.

Cloning of Hrs cDNA. According to the determined amino acid sequences, degenerate oligonucleotide primers were designed to amplify an Hrs cDNA fragment from B16-F1 poly $(A)^+$ RNA by reverse transcriptase-PCR. The amplification with primers corresponding to the sense and antisense sequences of the heptapeptide, VVQDTY and (K)FGIEK (we assumed the amino acid residue before F to be K, because the fragment was obtained by digestion with lysylendopeptidase), respectively, resulted in a fragment of 246 bp. This PCR product encoded 82 amino acids that contained three peptide sequences derived from purified Hrs in the same coding frame (VVQDTYQIMK, YSTIPK, and FGIEK in Fig. 2B). The PCR clone was used as a probe to screen a cDNA library from B16-F1 cells, and two clones, 2-1 (953 bp long, nucleotides 1 to 953) and 5-1 (1,079 bp long, nucleotides 266 to 1344), were obtained. Clone 2-2 (1,193 bp long, nucleotides 905 to 2097) was subsequently obtained by screening the library with *Pst*I-*Pst*I fragment (nucleotides 811 to 1200) of clone 5-1, and clone 1-2 (1,024 bp long, nucleotides 1435 to 2458) was further obtained by screening with *Sph*I-*Eco*RI fragment (nucleotides 1898 to 2097) of clone 2-2. Clones 2-1, 5-1, 2-2, and 1-2 were sequenced to determine the primary structure of mouse Hrs. The positions of the clones and the nucleotide sequence of the cDNA are shown in Fig. 2A and B, respectively.

Predicted amino acid sequence of Hrs. The amino acid sequence was deduced from the cDNA sequence (Fig. 2B). The translation initiation site was assigned to the first methionine codon (nucleotides 43 to 45) because the surrounding sequence GTCGCCATGG matches the favorable Kozak consensus sequence (35). Although there was no in-frame stop codon upstream of the methionine codon in the mouse sequence, it was found 45 bp upstream of the corresponding methionine codon of human Hrs cDNA (36a), further confirming that the first methionine codon is the translation initiation site. The open reading frame consisted of 775 amino acids, and the protein product had a calculated molecular weight of 86,014. No hydrophobic core region was found throughout the sequence, suggesting that Hrs is not an integral membrane protein.

A comparison of the predicted protein sequence of Hrs with sequences in the Swiss Prot and NBRF protein databases did not reveal the presence of any domains that would allow prediction of the function of Hrs. However, the sequence contained several characteristic features. The most interesting was a double zinc finger-like structural motif in the N-terminal region (Fig. 2B). This region showed the highest homology to five proteins from various organisms: the human faciogenital dysplasia gene product FGD1 (50) (42% homology), human early endosome-associated protein EEA1 (44) (48% homology), *Saccharomyces cerevisiae* protein Fab1 (Swiss Prot accession number P34756) (46% homology), *S. cerevisiae* protein N2038 (NBRF accession number S45129) (46% homology), and *Caenorhabditis elegans* protein ZK632.12 (Swiss Prot accession number P34657) (39% homology) (Fig. 3). In addition, a zinc finger domain in *S. cerevisiae* Vac1, a protein required for vacuole inheritance and vacuole protein sorting (69), was partially homologous (Fig. 3). However, the functions of the zinc finger-like domains in these proteins are not known at present. Other characteristic features included a proline-rich region (20 proline residues among 101 amino acids) and a proline- and glutamine-rich region (43 proline and 47 glutamine residues among 268 amino acids) in the middle and C-terminal portions of the protein, respectively (Fig. 2B).

There are 30 tyrosine residues in Hrs. Among these, tyrosines 132, 237, 394, and 522 were preceded by a negatively charged amino acid (aspartic or glutamic acids) and may serve as phosphorylation sites (28).

Hrs cDNA encodes the 115-kDa tyrosine-phosphorylated protein. To ensure that the Hrs cDNA encoded the 115-kDa tyrosine-phosphorylated protein, the open reading frame from the cDNA clone 5-1 was expressed as a fusion protein with glutathione-*S*-transferase in *E. coli* and a polyclonal antibody against the recombinant fusion protein was raised. When proteins from B16-F1 cells treated with or without HGF were immunoprecipitated by the antibody and subsequently immunoblotted by the same antibody, a single 115-kDa band was detected in both cells (Fig. 4A, left panel). Immunoblotting the same samples with PY20 showed that tyrosine phosphorylation of this protein was induced in cells treated with HGF (Fig. 4A, right panel). These results indicated that the cDNA encodes the 115-kDa protein which is tyrosine phosphorylated in HGF-treated cells.

To confirm that the cDNA contained the entire coding sequence, we performed a transient-expression experiment with Cos-7 cells. Because Cos-7 cells expressed a high level of Hrs (data not shown), we constructed an HA epitope-tagged Hrs expression vector to distinguish transfected Hrs from the endogenous protein. This vector expresses a protein (HA-Hrs) with an additional 20-amino-acid sequence, including the HA epitope before the N terminus of Hrs. Proteins from transfected Cos-7 cells were immunoprecipitated by the anti-HA

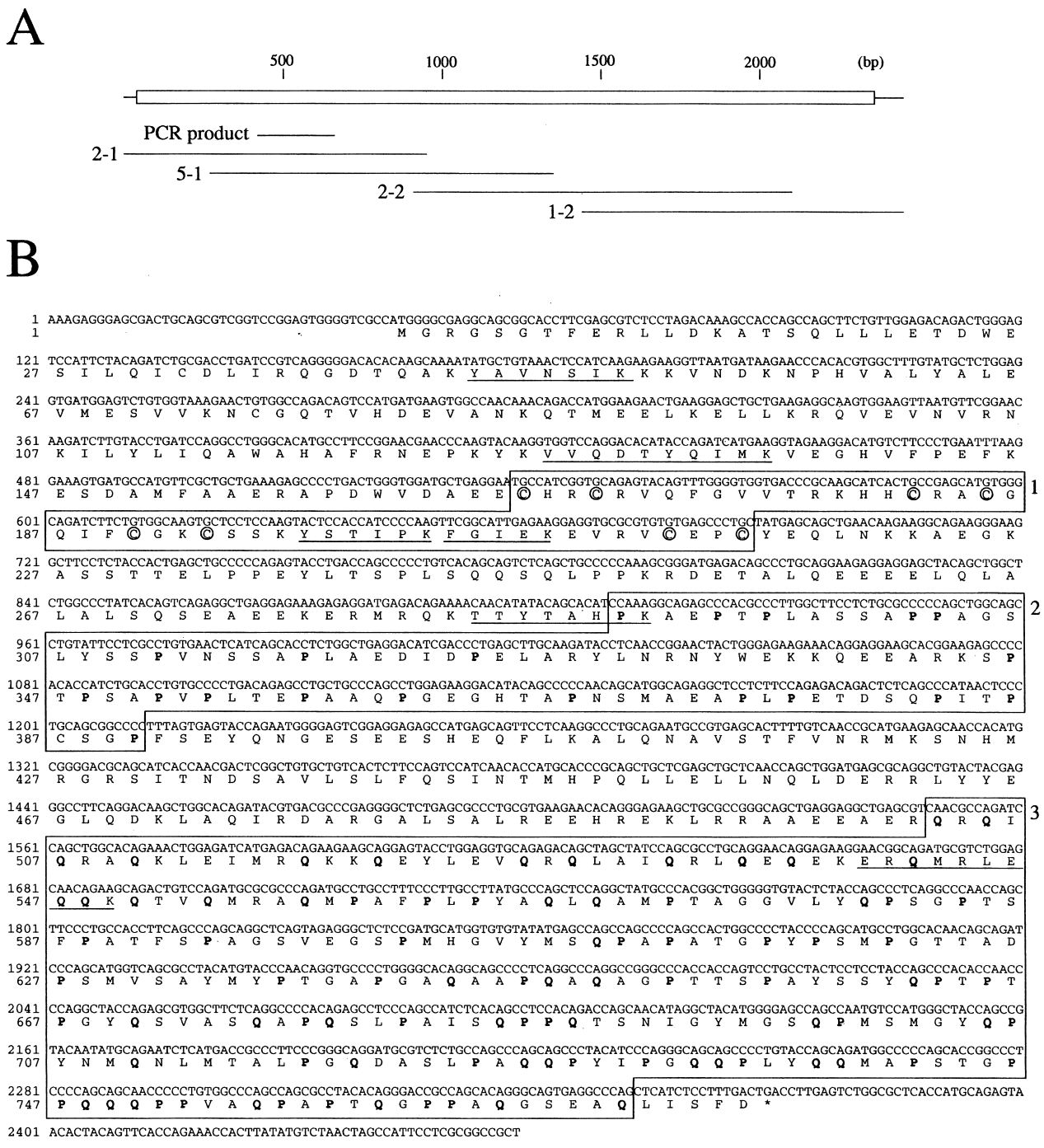

FIG. 2. Structure of Hrs cDNA. (A) Schematic representation of Hrs cDNA clones. The coding region is indicated by a box. (B) Nucleotide and predicted amino acid sequences of Hrs cDNA. The sequence of Hrs cDNA is shown and translated from the first ATG codon in the open reading frame. Nucleotide and amino acid numbers are shown at the left. Regions corresponding to the amino acid sequences of the peptides derived from purified Hrs are underlined. The zinc finger-like domain is indicated by box 1, and the cysteine residues in the domain are circled. Boxes 2 and 3 represent a proline-rich region and a proline- and glutamine-rich region, respectively. The proline residues in box 2 and the proline and glutamine residues in box 3 are shown in boldface type.

epitope antibody 12CA5, and the immunoprecipitates were immunoblotted with anti-Hrs. Two bands with apparent molecular masses of 116 and 90 kDa were detected (Fig. 4B, lane 2). These bands were not detected in Cos-7 cells transfected with a control vector lacking the HA epitope and the Hrs sequences (Fig. 4B, lane 3), indicating that these bands were both derived from HA-Hrs cDNA. Since HA-Hrs is 20 amino acids larger than Hrs, the appearance of the 116-kDa band, which is slightly larger than Hrs from B16-F1 cells (Fig. 4B, lane 1), indicated that the Hrs cDNA contains the entire coding region. The smaller 90-kDa protein may be a degradation product, probably as a result of overexpression in the transfected cells or instability of the protein caused by HA tagging. Alternatively, it is possible that the 90-kDa protein is a pre-

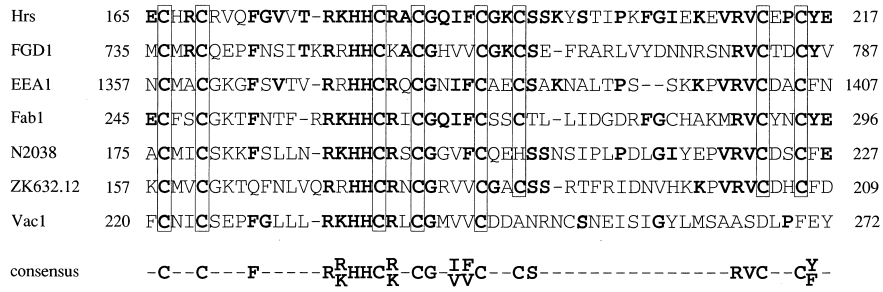

FIG. 3. Alignment of the amino acid sequence of the zinc finger domain of Hrs with that of the corresponding regions of FGD1, EEA1, Fab1, N2038, ZK632.12, and Vac1. The conserved cysteine residues (and a histidine residue in N2038) are boxed, and the residues common to Hrs are shown in boldface type.

cursor of Hrs that accumulated in the transfected cells as a result of overexpression, because the apparent molecular mass is quite similar to the calculated molecular mass of the Hrs cDNA product (86 kDa).

Characterization of Hrs tyrosine phosphorylation. We characterized the tyrosine phosphorylation of Hrs in B16-F1 cells by using anti-Hrs. Figure 5A shows the time course of Hrs phosphorylation, which was evident within 2.5 min after HGF addition. It reached a maximal level at 5 min and then was reduced almost to the basal level within 2 h. On the other hand, the amount of Hrs was unchanged during incubation with HGF (Fig. 5A, lower panel).

To determine the maximal phosphorylation ratio of Hrs, the tyrosine-phosphorylated form of this protein was immunoprecipitated by PY20 from the lysate of cells incubated with HGF for 5 min (Fig. 5B, lane 1 in each panel). Unphosphorylated Hrs which was not absorbed to PY20 was then recovered from the supernatant by immunoprecipitation with anti-Hrs (lane 2 in each panel). This fraction did not contain the phosphorylated form as judged by immunoblotting with PY20 (Fig. 5B, right panel). Phosphorylated and unphosphorylated forms were detected by immunoblotting with anti-Hrs (Fig. 5B, left panel). Densitometric scanning of the band intensity showed that about 13% of the total Hrs pool was phosphorylated on tyrosine in HGF-treated cells.

Intracellular localization of Hrs. We determined the intracellular localization site of Hrs by subcellular fractionation. Proteins from cytoplasmic, membrane, and nuclear fractions of HGF-treated or untreated B16-F1 cells were immunoprecipitated with anti-Hrs and immunoblotted with the same antibody. Hrs was localized to the cytoplasm both in HGF-treated and untreated cells (Fig. 6A, top panel). Immunoblotting with PY20 revealed the tyrosine-phosphorylated form of Hrs, also in the cytoplasm (Fig. 6A, second panel from top). In contrast, the c-Met receptor, a cell surface transmembrane protein, and CREB, a transcription factor which is localized solely to the nucleus (17), were recovered in the membrane and nuclear fractions, respectively, verifying the integrity of each fraction (Fig. 6A, third and bottom panels, respectively).

Some receptor tyrosine kinase substrates form stable complexes with autophosphorylated receptors. We therefore determined whether Hrs binds stably to the HGF receptor tyrosine kinase, c-Met. Anti-Met and anti-Hrs did not coprecipitate Hrs

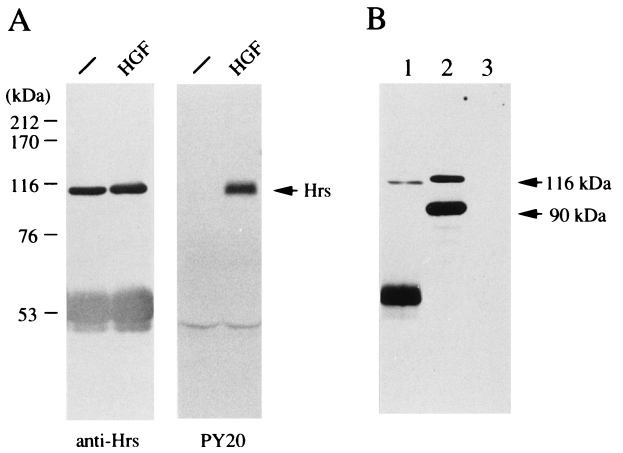

FIG. 4. Detection of Hrs by anti-Hrs polyclonal antibody. (A) B16-F1 cells were incubated without (−) or with 100 ng of HGF per ml for 5 min, lysed, and immunoprecipitated with anti-Hrs. The immunoprecipitates were separated by SDS-PAGE under reducing conditions and immunoblotted with anti-Hrs or PY20. (B) Cos-7 cells transfected with the HA-Hrs expression vector (lane 2) or a control vector (lane 3) were lysed, immunoprecipitated with anti-HA epitope antibody 12CA5, and immunoblotted with anti-Hrs. Hrs immunoprecipitated from B16-F1 cells by anti-Hrs is shown as a molecular mass standard (lane 1).

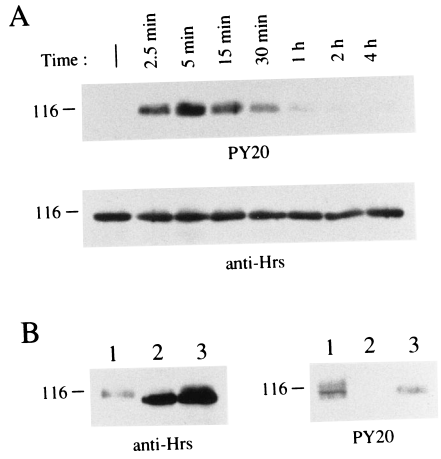

FIG. 5. Characterization of Hrs tyrosine phosphorylation. (A) Time course of Hrs tyrosine phosphorylation. B16-F1 cells were incubated with 100 ng of HGF per ml for the indicated periods, lysed, and immunoprecipitated with anti-Hrs. The immunoprecipitates were separated by SDS-PAGE under reducing conditions and immunoblotted with PY20 or anti-Hrs. (B) Tyrosine phosphorylation ratio of Hrs. Tyrosine-phosphorylated Hrs was immunoprecipitated with PY20 from B16-F1 cells treated with 100 ng of HGF per ml for 5 min (lane 1). Unphosphorylated Hrs which was not absorbed to PY20 was then recovered from the supernatant by immunoprecipitation with anti-Hrs (lane 2). Total Hrs was independently immunoprecipitated from the lysate of HGF-treated cells with anti-Hrs (lane 3). The immunoprecipitates were separated by SDS-PAGE under reducing conditions and immunoblotted with anti-Hrs or PY20.

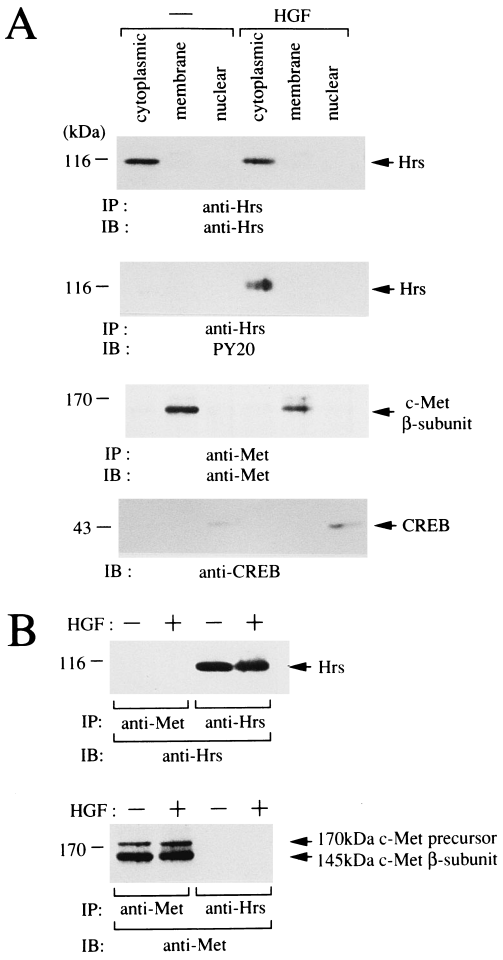

FIG. 6. Intracellular localization of Hrs. (A) Subcellular fractionation of Hrs. B16-F1 cells incubated without (−) or with 100 ng of HGF per ml for 5 min were homogenized and fractionated into cytoplasmic, membrane, and nuclear fractions as described in Materials and Methods. Proteins from each fraction were extracted, immunoprecipitated with anti-Hrs or anti-Met, and immunoblotted with the indicated antibodies. Otherwise, the proteins were directly immunoblotted by anti-CREB. (B) No association of Hrs with c-Met receptor. B16-F1 cells were incubated without (−) or with (+) 100 ng of HGF per ml for 5 min, lysed, and immunoprecipitated with anti-Hrs or anti-Met. The immunoprecipitates were separated by SDS-PAGE under reducing conditions and immunoblotted with anti-Hrs or anti-Met. IP and IB represent immunoprecipitation and immunoblotting, respectively.

and c-Met, respectively, in either HGF-treated or untreated cells (Fig. 6B), indicating that these proteins do not form a stable complex at least under our experimental conditions. These results are consistent with the cytoplasmic localization of Hrs even when tyrosine phosphorylated.

Tyrosine phosphorylation of Hrs by other growth factors. To examine whether tyrosine phosphorylation of Hrs is specific for HGF stimulation, we tested the phosphorylation of Hrs in cells treated with other growth factors. In A431 human epidermoid carcinoma cells, both HGF and epidermal growth factor induced the tyrosine phosphorylation of Hrs (Fig. 7). Furthermore, treatment of NIH 3T3 mouse fibroblasts with platelet-derived growth factor BB also induced Hrs phosphorylation (Fig. 7). These results indicated that Hrs is a common substrate for several receptor tyrosine kinases or that several growth factors activate an intracellular tyrosine kinase that phosphorylates Hrs. The electrophoretic mobilities of Hrs from EGF-treated and HGF-treated A431 cells were slightly different on

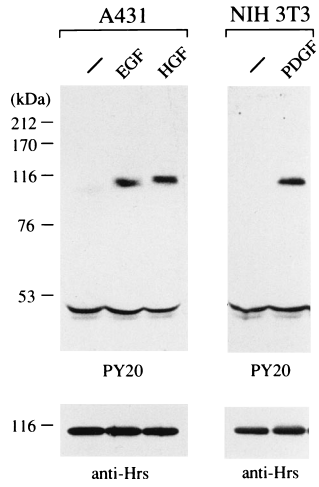

FIG. 7. Tyrosine phosphorylation of Hrs by other growth factors. A431 and NIH 3T3 cells, cultured for 24 h in Dulbecco's modified Eagle's medium supplemented with 0.5% fetal calf serum, were incubated without (−) or with 100 ng of the indicated ligands per ml for 5 min, lysed, and immunoprecipitated with anti-Hrs. The immunoprecipitates were separated by SDS-PAGE under reducing conditions and immunoblotted with PY20 or anti-Hrs.

SDS gels (Fig. 7). Therefore, it is possible that the phosphorylation sites of Hrs are different in these cells. Alternatively, there may be two related proteins with similar molecular masses which are recognized by anti-Hrs.

Tissue distribution of Hrs mRNA. We determined the size and tissue distribution of Hrs mRNA by Northern blotting with poly(A)$^+$ RNAs from various mouse tissues. A single transcript of 3.0 kb was detected in a variety of mouse adult tissues (Fig. 8A). Among them, the expression level of Hrs mRNA was highest in the testis (Fig. 8A). It was also detected in poly(A)$^+$ RNA preparations from various stages of mouse whole embryos (Fig. 8B).

DISCUSSION

In this study, we identified a novel tyrosine kinase substrate in growth factor-treated cells. This protein, Hrs, consists of 775

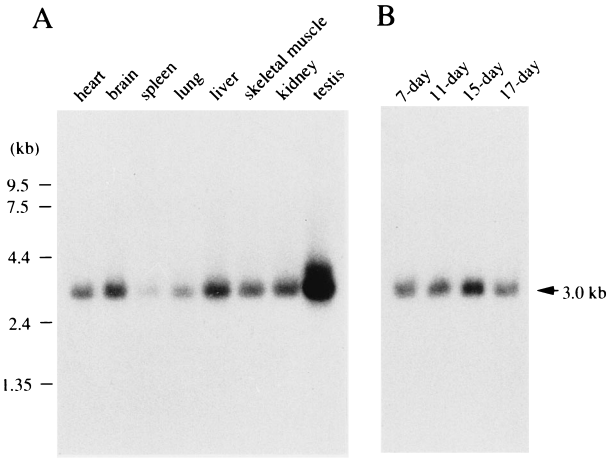

FIG. 8. Northern blot analysis of RNAs from various mouse tissues. Poly (A)$^+$ RNAs (2 μg) from various adult mouse tissues (A) and various stages of mouse whole embryos (B) were analyzed with Hrs cDNA as a probe. Size markers are indicated in kilobases.

amino acids with a calculated molecular mass of 86 kDa. The apparent molecular mass of Hrs detected in cultured cells and of recombinant Hrs expressed in Cos-7 cells was about 115 kDa, as determined by SDS-PAGE. Thus, this protein may migrate somewhat more slowly on SDS-polyacrylamide gels than its calculated molecular mass. Alternatively, this may be due to some unknown posttranslational modifications of the protein.

Tyrosine phosphorylation of Hrs was induced rapidly after HGF treatment and then rapidly decreased. This suggested that Hrs plays a role at an early stage of the HGF signaling cascade and that the activity of Hrs is strictly regulated by a protein tyrosine phosphatase. In addition, Hrs mRNA was expressed in a variety of adult mouse tissues and also in embryos, and the tyrosine phosphorylation of Hrs was induced by several growth factors. These results, together with the presence of a zinc finger-like structural motif which is conserved from yeasts to higher vertebrates, suggest an important function of Hrs in the signaling pathway of various growth factors in a variety of cell types.

The zinc finger domain of Hrs showed the highest sequence homology to five zinc finger proteins. They included the human faciogenital dysplasia gene product FGD1, human early endosome-associated protein EEA1, yeast Fab1 protein, yeast N2038 protein, and nematode ZK632.12 protein. Among these proteins, FGD1 has a domain structurally conserved in the Rho/Rac guanine nucleotide exchange factors and is considered to function as a guanine nucleotide exchange factor for a small GTP-binding protein(s) of the Ras family (50). EEA1 is a hydrophilic peripheral membrane protein associated with early endosomes and is proposed to play a role in vesicular transport of proteins through early endosomes (44). However, the role of the zinc finger domains of these proteins remains unknown. In addition, the functions of the other proteins are unknown. Although the function is unclear, the high sequence homology in the zinc finger domains, which is conserved from yeasts to humans, suggests that these domains form a new subfamily of zinc finger structures with the same or a similar biologically important function.

Zinc finger domains are binding sites for various macromolecules. A number of DNA- or RNA-binding proteins possess zinc finger domains and bind to the specific nucleotide sequences through these structures (30). Many other proteins also have zinc finger domains. There is a subfamily of a structurally conserved zinc finger-like motif in several proteins including protein kinase C. Among these, the zinc finger domains in protein kinase C (47), the *unc-13* gene product (38), *n*-chimaerin (1), and Vav (20) are shown to be binding sites for phorbol esters or diacylglycerol, and the binding of these molecules activates the enzymatic activities of the proteins. The LIM domain is another structurally conserved subfamily of zinc finger structures (58). Recently, it was reported that the LIM domains in the cysteine-rich protein and zyxin function as protein-protein interaction motifs (13, 59). Considering the fact that Hrs was detected in the cytoplasm but not in the nucleus, the zinc finger domain in Hrs may mediate interactions with other proteins or lipid mediators rather than function as a DNA-binding domain. The absence of a typical nuclear localization signal in the Hrs sequence and the presence of a homologous zinc finger structure in a putative guanine nucleotide exchange factor, FGD1, and an early endosome-associated protein, EEA1, support the notion that it is not a DNA-binding protein.

Other characteristic structures in Hrs are a proline-rich region and a proline- and glutamine-rich region that reside in its middle and C-terminal regions, respectively. Although the functions of proline-rich regions are often unclear, there are some examples showing that it is involved in multiple protein interactions. Some transcription factors contain a proline-rich sequence in the transcriptional activation domain, which has no DNA-binding activity but is essential for activating transcription (40). The proline-rich transcriptional activation domain in CCAAT box-binding transcription factor 1 is shown to directly contact transcription factor IIB to form a transcription initiation complex (29). A glutamine-rich region is also present in the transcriptional activation domains of some transcription factors (40). The glutamine-rich regions in Sp1 and *fushi tarazu* are shown to bind a protein component of the transcription initiation complex (6, 22). Another example is the SH3 domain-binding motif. It consists of a proline-rich peptide of about 10 amino acids with a core consensus sequence, XPXPPXP, in which the proline residues are essential for high-affinity binding to SH3 domains (54, 71). SH3-binding motifs are found in several proteins involved in cell growth signaling, and these proteins form signaling complexes by binding to SH3-containing proteins (51). However, despite the presence of proline-rich regions, the SH3-binding consensus sequence was not found in Hrs, suggesting that Hrs is not an SH3-binding protein.

SH2 and SH3 domains are also often found in tyrosine-phosphorylated proteins involved in cell growth signaling and play roles in protein-protein interactions to form signaling complexes (31, 51). These domains were not found in Hrs. The absence of SH2 domains in Hrs is consistent with the fact that the association of Hrs with the autophosphorylated c-Met receptor could not be detected in living cells. On the other hand, it is possible that phosphorylated tyrosine residues in Hrs serve as binding sites for some SH2 domain-containing proteins. The SH2 domain-binding specificity of each phosphotyrosine is determined by the surrounding sequence, usually three residues immediately following the phosphotyrosine (60). There are some tyrosine-containing sequences which may bind to proteins containing SH2 domains in Hrs. The sequences including tyrosines 132 (YQIM), 617 (YPSM), and 737 (YQQM) match the consensus sequence of the binding site for the SH2 domains of the phosphatidylinositol 3′-kinase p85 subunit (YXXM) (4, 10, 60). The sequence including tyrosine 522 (YLEV) is a favorable binding site for the N-terminal SH2 domain of phospholipase C-γ1 (60). Therefore, identifying the tyrosine phosphorylation sites of Hrs will help identify the proteins containing SH2 domains that may associate with Hrs.

Considering these structural features, it is possible that Hrs is associated with other molecules through the zinc finger domain, the proline-rich region, the proline- and glutamine-rich region, and/or the probable SH2-binding sites containing phosphotyrosine and that Hrs plays a role in regulating the activities of the associated proteins which may be important for growth factor signaling. However, it is also necessary to consider the possibility that Hrs has an unknown enzyme activity, which is important for the signaling pathway mediated by Hrs.

To understand the function of Hrs, it is necessary to identify the molecules that interact with it. It is not known at present whether Hrs is a direct substrate for growth factor receptors. In addition to the fact that the association between Hrs and c-Met receptor was undetectable in living cells, Src family tyrosine kinases are activated in cells treated with growth factors including HGF (8, 42, 53). Therefore, it is also possible that an intracellular nonreceptor tyrosine kinase phosphorylates Hrs. Further experiments are required to identify the tyrosine kinase which is responsible for the phosphorylation of Hrs. Moreover, and much more importantly, the structure of Hrs suggests that it may associate with other proteins. Identifica-

tion of the proteins that associate with Hrs will clarify the role of Hrs in the signaling pathway triggered by growth factors.

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